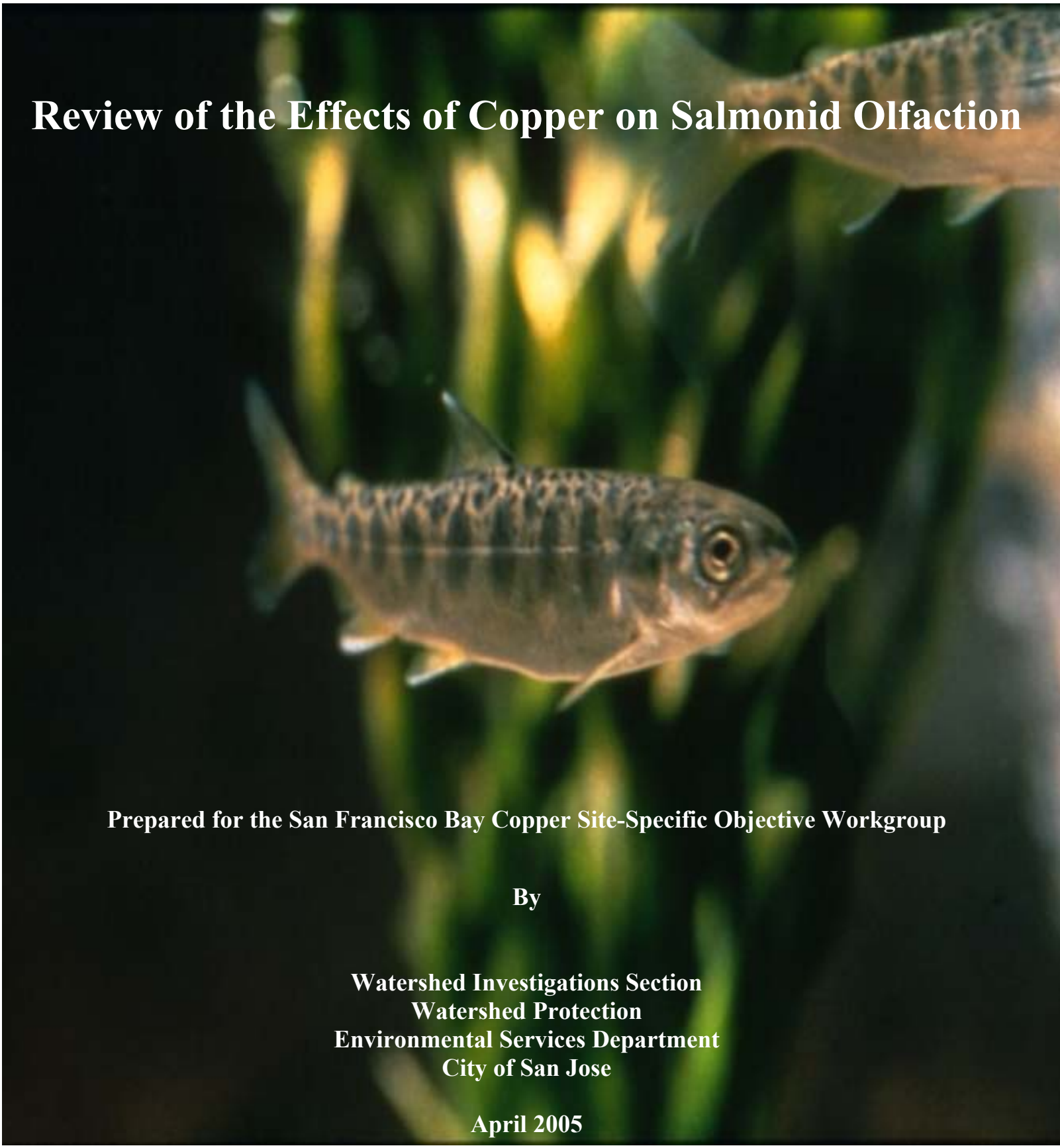
Review of the Effects of Copper on Salmonid Olfaction



Prepared for the San Francisco Bay Copper Site-Specific Objective Workgroup

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Juvenile coho salmon photographed by Desmond Maynard courtesy of NOAA Fisheries, Seattle, WA

Executive Summary

The efforts in San Francisco Bay, both north and south of the Dumbarton Bridge, to assess the effects of copper have concluded that impairment of beneficial uses in the Bay due to copper is unlikely. However, the National Marine Fisheries Service (NOAA Fisheries) recently asked the Water Board staff to consider an examination of the sublethal effects of copper on salmonid olfactory function as part of the triennial review process and the north of Dumbarton Bridge (NDB) copper site-specific water quality objective (SSO) development effort. This review summarizes the effects of copper on salmonid olfaction, as reported in the literature, based on freshwater laboratory tests. It assesses the ecological relevance of available study results to the San Francisco Bay and evaluates the protectiveness of the proposed site-specific objectives of 6.0 and 6.9 μg/L for the southern/lower and central/northern portions, respectively, of San Francisco Bay.

Olfaction plays a key role in species recognition, migration, reproduction, and predator avoidance in salmonids. Twenty-five neurophysiological and neurobehavioral endpoints from nine freshwater studies were examined. Results indicate that salmonids are sensitive to copper, with endpoints ranging from 0.8 – 200 μg/L. A comparison of the olfactory endpoints to the current EPA hardness-based copper criteria for freshwater indicates that most endpoints (80%) are protected by the current criteria. One limitation of these studies is that all nine used well or municipal water sources that were low in organic carbon compared to California natural streams (< 2 vs. 9.33 mg/L). Therefore, the olfactory results reported are very likely over-protective of natural stream environments.

To illustrate the potential effect of dissolved organic carbon (DOC) on test endpoints, the Biotic Ligand Model (BLM) was used to extrapolate the copper effect levels from the conditions in the most sensitive test, which had an endpoint of 0.8 μg/L, to conditions that are more representative of natural waters. The test water (diluted well water) contained approximately 0.03 mg C /L as DOC. The BLM predicted that the 0.8 μg/L dissolved copper effect level observed would have approximately equated to a 34 μg/L dissolved copper concentration in test water with a DOC of 2 mg C/L. An assessment of the ecological relevance of the test results is difficult to make because all nine studies did not consider the organic content of natural waters.

A search of the available literature did not reveal any studies on the effects of copper on salmonid olfaction in seawater. Nevertheless, a comparison can be made between study results in freshwater and the San Francisco Bay based on the concentration of free ionic copper (Cu^{2+}), since the toxicity of copper to aquatic organisms is due mainly to Cu^{2+}, the bioavailable fraction of the metal. Using the BLM, the Cu^{2+} concentration from the most sensitive test (0.8 μg/L copper) can be back calculated as 4.4×10^{-10} M. This concentration is three orders of magnitude greater than the Cu^{2+} concentration of 10^{-13} M measured as several locations throughout the Bay (Buck & Bruland 2005). In other words, Cu^{2+} concentrations in the Bay would have to increase more than 1,000 times before reaching levels that may inhibit olfaction in Chinook salmon, the species identified in the present literature review whose olfactory function was found to be the most sensitive to copper.

There has been no measurable change in Lower South Bay ambient dissolved copper concentrations since adoption of the 6.9 μg/L SSO in 2002. Similarly, there is no expectation that there would be ambient increases NDB solely due to adoption of the proposed 6.9 and 6.0 μg/L SSOs. Ambient copper monitoring and source control measures will be implemented as part of the concurrently adopted Copper Management Strategy to help ensure continued protection of beneficial uses.

Introduction

The "Impairment Assessment Report for Copper and Nickel in Lower South San Francisco Bay" (Tetra Tech 2000) and the "North of Dumbarton Bridge Copper and Nickel Conceptual Model and Impairment Assessment Report" (EOA, Inc. & Larry Walker Associates 2004) both concluded that impairment of beneficial uses in the Bay due to copper and nickel is unlikely. In June 2004, draft copper SSOs of 6.4 and 6.9 μg/L were recommended by the workgroup (the latter was identical to the copper SSO adopted in 2002 for the Lower South Bay). On June 17, 2004 the National Marine Fisheries Service (NOAA Fisheries) submitted a comment letter on the Basin Plan Triennial Review process. One of the comments in the letter requested that the Water Board staff consider an examination of the sublethal effects of copper on salmonid olfactory performance as part of the triennial review process and the NDB SSO effort.

City of San Jose staff prepared the present literature review of laboratory studies on the effects of copper on salmonid olfactory function in fresh water. This review examines the current scientific research on this subject relevant to the appropriateness of the recommended SSOs for the Bay. This review summarizes: 1) the role of olfaction in salmonids, 2) the methods for obtaining electro-olfactograms (EOGs) in fish, 3) the potential inhibitory nature of copper on salmonid peripheral olfaction as measured by the EOG and other methods, 4) current research on copper-mediated behavioral responses in fish, 5) the relationship of EPA and site-specific copper criteria to copper/olfactory effects levels, and 6) essential elements for establishing ecological relevance of copper/olfactory study results derived in freshwater to San Francisco Bay/Estuary.

The Role of Olfaction in Salmonids

The role of salmonid olfaction in predator avoidance, sibling and species recognition, migration, and reproduction has been studied extensively. Some 50 years ago, researchers found that upstream migration of Pacific salmon was drastically reduced, from a mean rate of 34 to 4 fish per 10-minute interval, when human hands were immersed in upstream water (Brett and MacKinnon 1952). Subsequent research confirmed that the human repellent caused an alarm reaction characterized by rapid movements, confinement of fish to pools, retreat downstream and a keen alertness during which time slight disturbances of the water would cause an immediate response (Idler *et al*. 1956). This work identified the active ingredient in the repellent as L-serine and acknowledged that this amino acid repelled all five species of migrating salmon found in the Pacific Northwest (coho, spring, chum, sockeye and pink). Other components of skin, including phosphate esters, sugars, fatty acids, and purine and pyrimidine compounds did not activate the alarm response. In contrast, L-Serine was shown to have repellent effects at concentrations as low as "one part in several million parts of water" (Idler *et al*. 1956).

Juvenile rainbow trout exhibit an alarm response when exposed to skin extract from conspecifics (other rainbow trout) but not to trout body extract (without skin) or to swordtail skin extract (Brown & Smith 1997). The antipredator behavior exhibited by trout exposed to the putative (supposed) alarm pheromone included freezing, reduced time spent swimming, and increased time to resume feeding (Brown & Smith 1997). However, when the chemical cues come from water conditioned by fish rather than from fish skin extract, the exposed salmonids may not respond. For example, Arctic charr (*Salvelinus alpinus*) prefer water conditioned by similar-sized conspecifics and other salmonids, but clearly avoided water with predator (pike or burbot) odors (Hirvonen *et al*. 2000). Young charr more readily approached the stimulus source when the water was from trout fed on

pellets than when it was from trout fed on charr, indicating that charr could detect an unknown chemical stimulus (Hirvonen *et al*. 2000).

Kin recognition in salmonids was demonstrated by Quinn & Busack (1985), who found that juvenile coho salmon (*Oncorhynchus kisutch*) preferred water conditioned by both familiar and unfamiliar siblings over non-siblings. Since salmonid taste receptors are very sensitive to bile acids, it cannot be assumed that this chemosensory recognition was due entirely to olfaction (Quinn & Busack 1985). Nevertheless, olfaction appears to play a major role.

One of the most well known olfactory-mediated behaviors of salmonids is their homing ability. The role of olfaction in salmonid homing was successfully field tested in 1954 (Wisby and Hasler 1954). Salmon inhabiting the Atlantic (*Salmo salar*) and Pacific (*Oncorhynchus keta, O. kisutch, O. nerka, O. gorbuscha, and O. tschawytscha*) spawn and rear in freshwater, migrate to saltwater and remain there until sexually mature, and then return home to their natal streams with remarkable accuracy (Kleerekoper 1969). Early studies of salmonid homing found that:

1) olfaction was necessary for correct homing
2) each stream has a characteristic and persistent odor that salmon can distinguish
3) salmon could be artificially "imprinted" using organic compounds such as morpholine, a heterocyclic amine, and phenethyl alcohol (PEA)
4) imprinting occurs when fish are about 18 months old at the time they undergo transformation (smoltification) from parr to smolt
5) salmon could be imprinted in as little as two days
6) the presence of home odors elicits positive rheotaxis in salmon (i.e. swimming upstream when the odor is present; backtracking downstream when the home odor is absent)
7) the sensitivity of salmon to home stream odors increases over the spawning season due to increasing sex hormones
8) amino acids are effective olfactory stimuli (see reviews by Hara 1975, and Hasler & Scholz 1983).

Seemingly contradictory homing experiments have shown that fish odors are necessary (Doving 1996) and not necessary (Brannon *et al.* 1984) for home water recognition. Salmon appear to sense differences in the amino acid composition of streams to successfully return to their natal stream (Shoji *et al*. 2000). This seems to make sense since migrating salmon often have to distinguish among several potential home streams that may vary little in the amounts and types of amino acids they contain. In a review of available literature, observations, and field studies, Brannon (1981) concluded that homing behavior of Pacific salmon appears to be a response to odors related to the environmental chemistry of a particular habitat, and that if fish odors are a part of the "bouquet" they are decisive only when other environmental cues are undifferentiated.

A review by Liley (1996) indicated that olfaction plays a prominent role in salmonid reproduction. For example, studies have found that pheromones appear to play both releasing (triggers behavioral responses) and priming (induces physiological responses without any immediate change in behavior) roles in salmonid reproduction. The priming response depends on olfaction while visual and other cues do not compensate for the lack of olfactory input. However, maintenance of spawning behavior does **not** depend on olfaction. Both male and female rainbow trout respond positively to water taken downstream of spawning conspecifics. Furthermore, male rainbow trout will court preovulatory females following the addition of ovarian fluids to the holding tank, and plugging the nares of males eliminated the male courtship response. Finally, spermiating males that were rendered anosmic by

cauterization of the olfactory epithelium did not have any increase in milt and actually had a decline in plasma concentrations of gonadal steroids, even though paired with nesting females for three days.

Exposure of mature male Atlantic salmon (Salmo salar) parr to waterborne F-type prostaglandins, $PGF_{1\alpha}$ & $PGF_{2\alpha}$, at 10^{-8} mol l^{-1}, resulted in significant increases in levels of expressible milt and plasma hormone concentrations (Moore & Waring 1996a). Urine of female Atlantic salmon contained large quantities (18 ng/ml) of immunoreactive PGFs, while that of mature males and non-ovulated females contained significantly smaller amounts (<1ng/ml). Behavioral and olfactory (electro-olfactogram or EOG) experiments on brown trout and lake whitefish exposed to $PGF_{2\alpha}$ also indicated that this compound functions as a reproductive pheromone in salmonids (Laberge & Hara 2003).

The Use of Electro-olfactograms in Measuring Salmonid Olfactory Function

Electrical responses of the olfactory epithelium of salmonids have been the subject of numerous investigations over the past 35 years (Sutterlin & Sutterlin 1971; Stacey *et al*. 1996; Baldwin *et al*. 2003; Sandahl *et al*. 2004). Electrical responses have been used to study: 1) salmonid olfactory responses to different compounds and solutions (Sutterlin & Sutterlin 1971; Hara 1972); 2) essential salmonid activities and behaviors, including reproduction (Laberge & Hara 2003) and thyroid function and smoltification (Morin *et al*. 1997); and 3) the effects of pollutants, such as mercury (Baatrup *et al.* 1990), acidification (reviewed in Moore and Waring 1996b), copper (Baldwin *et al.* 2003), and pesticides (Sandahl *et al.* 2004) on olfaction in salmonids.

Neurological responses of salmonid fish to natural odors and to a variety of toxicants can be studied using three key methods, electroencephalogram (EEG), electro-olfactogram (EOG), and brain acetylcholinesterase (AChE) activity. An EEG records an electrical response from the olfactory bulb (forebrain). An EOG records an electrical response from the peripheral olfactory epithelium in the fish nares (nose). AChE is the enzyme that hydrolyzes the neurotransmitter, acetylcholine. Measurements of brain AChE in test animals can be compared to that in control animals to determine the amount of inhibition of normal brain neurotransmission. The present discussion focuses primarily on the EOG and its use in measuring salmonid olfactory function.

Scott and Scott-Johnson (2002) have recently reviewed the history and uses of the EOG. EOGs have become an established technique for studying peripheral olfaction in fish as a result of the work of many researchers (e.g. Sutterlin & Sutterlin 1971; Bjerselius *et al*. 1993). Recently, Baldwin *et al.* (2003) and Baldwin & Scholz (in Press) refined and updated the methods for obtaining EOGs. The following discussion is a brief summary of the methods contained in these two publications (unless otherwise noted) and the work of the Northwest Fisheries Science Center researchers, under the direction of Dr. Nathaniel Scholz. A more detailed description of these methods can be found in the referenced reports (Baldwin *et al.* 2003 and Baldwin & Scholz, in Press).

The peripheral olfactory system in salmonids consists of a pair of olfactory rosettes that are located on the "nose" of the fish inside chambers that are covered with skin. The rosettes are so named because they are round and contain several folds of lamellae covered with sensory epithelium. The epithelium contains olfactory receptor neurons (ORNs) that are in direct contact with the aquatic environment. Environmental odors bind to the apical cilia or mocrovilli of ORNs, thus setting up an action potential. Thommesen (1983) showed that, in salmonids, the microvillar ORNs respond to amino acids and the ciliated ORNs respond to bile acids. ORN axons project to the olfactory regions of the brain. The EOG is measured at the rosette and its amplitude corresponds to the sum of the

electrical responses of many ORNs as they bind to dissolved odors. The EOG is an electrical field potential that takes place outside of the cell and it consists of a large, negative voltage transient (electrical current) measured with an electrode placed near the surface of the sensory epithelium of the rosette.

The standard EOG recording method, which can be used to record responses from salmonids (and other fishes) to a wide variety of odors and neurotoxicants, is shown in Figure 1. Stock solutions of odorants or neurotoxicants are prepared weekly and the odorant stimulus solutions are prepared daily by diluting the stock into the background water (e.g. filtered, de-chlorinated municipal water). Electrodes are prepared daily by filling them with a 2% agar-saline solution. The microelectrodes are bridged to Ag/AgCl electrodes by 3M KCl and connected to the electronics prior to testing. Care is taken to use fish raised in uncontaminated hatchery (or other) water. Fish are anesthetized with MS-222 (50 mg/L final concentration) for about 20 minutes. This is usually sufficient to immobilize the fish but still allow opercular movement (breathing). The fish is then injected with 1 mL (0.3 mg) of paralytic gallamine triethiodide per kg of body weight. This is injected into the muscle tissue in several places on the sides of the fish. The anesthesia and paralytic insure that the fish remains motionless during EOG recording. The use of control fish ensures that these treatments have no effect on the EOG.

Fish are placed on a vibration table in a Plexiglas holder that contains additional supports and foam to keep the immobilized fish upright (Figure 1A). Chilled water is delivered to the holding tank, and, through a separate tube, to the mouthpiece. The mouthpiece, which is inserted into the fish's mouth just in front of the gill arches, delivers water (containing MS-222) to the gills at the rate of 120 mL per minute. To improve gill oxygenation, the operculum may be partially removed and a piece of foam used to further open the remaining part of the operculum. A wet towel is placed over the fish to keep it moist.

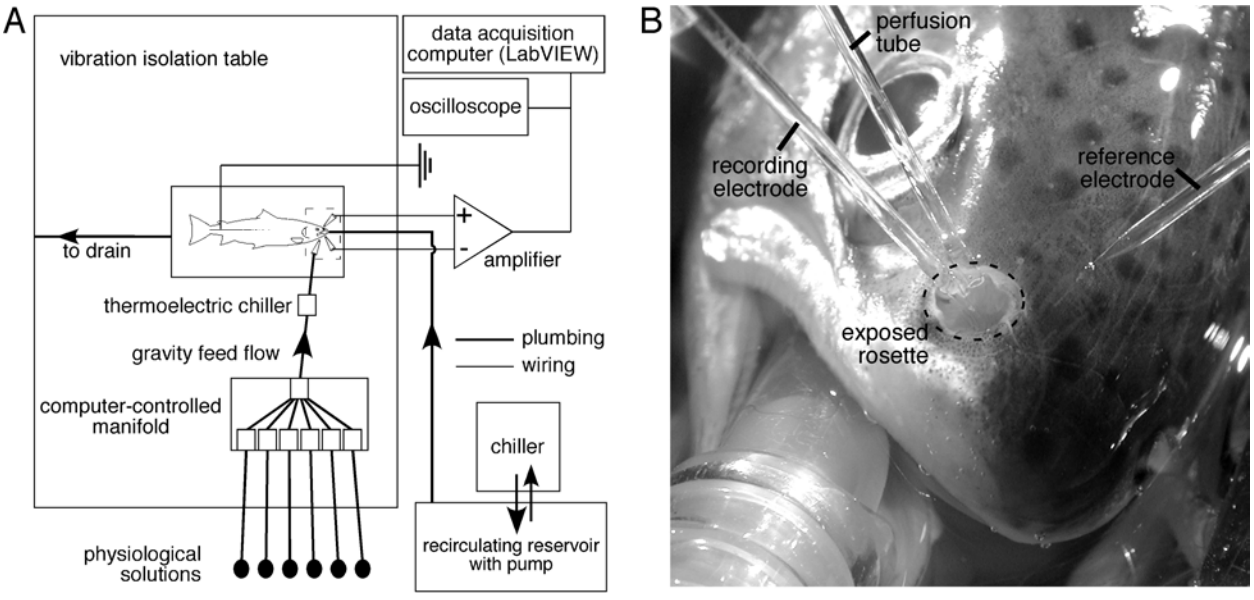


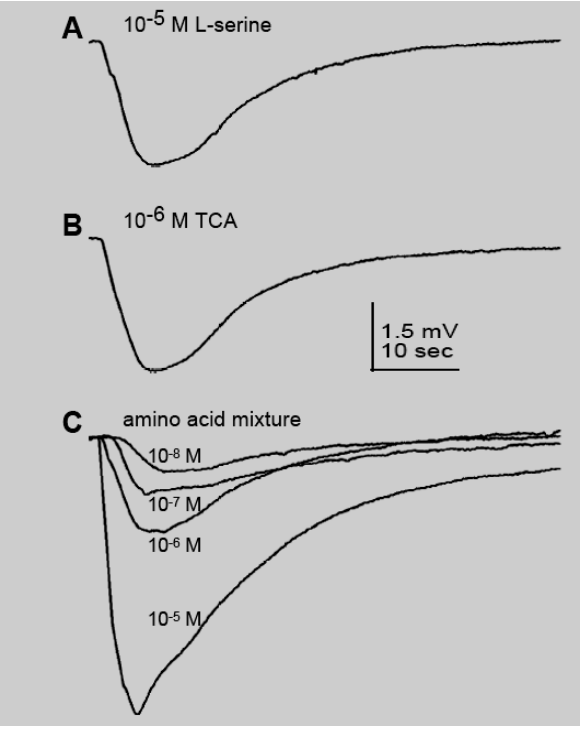
Figure 1. EOG recording system. A. Diagram of the major components of the apparatus. B. Juvenile coho salmon showing: 1) the mouthpiece providing water to the gills, 2) rosette with the skin removed, 3) recording electrode, 4) reference electrode, and 5) perfusion tube. Adapted from Baldwin *et al*. 2003. Diagram and photograph courtesy of NOAA Fisheries, Seattle WA.

A single olfactory rosette is exposed by removing the skin. Care must be taken to minimize bleeding, as the loss of blood to the rosette will result in the loss of olfactory response. A stereomicroscope and micromanipulator are used to place the recording microelectrode along the center of the rosette near the base of the posterior lamella. The reference electrode is placed into the skin of the rostrum behind (dorsal to) the rosette (Figure 1B). An additional grounding electrode (e.g. hypodermic needle) is inserted into the muscle near the tail (Figure 1A).

Fish are allowed to acclimate to test conditions for 15 minutes following placement of the electrodes. The delivery system (manifold in Figure 1A) is able to dispense control (background) water or test water (background water with odorant solutions and/or neurotoxicants) through two separate lines to the perfusion tube placed over the rosette (Figure 1B). Water is delivered to the exposed rosette at the rate of 5 mL/minute. Type and strength of the perfused odorant, pulse duration and interval all affect the (negative) amplitude of the electro-olfactogram. Thus, optimum responses from each species and lot of fish are determined empirically prior to each new experiment. A typical response "blank" or control EOG response is obtained by alternately pulsing control water through the dedicated control line and then through the line delivering odorants.

A computerized system delivers odorants to the olfactory rosette at a pre-set rate and duration, depending upon the results from the pre-test (e.g. a 10-second pulse of odorant followed by a 2-minute delivery of control water prior to the next pulse). If the odorant pulse is not long enough, the maximum response will not be obtained. If the inter-pulse interval is not long enough, subsequent pulses may decline due to adaptation. A typical EOG is shown in Figure 2.

Figure 2. Typical EOG recordings of an amino acid (**A**), a bile acid (**B**) and an amino acid mixture (**C**). Adapted from Baldwin *et al*. 2003.



To test a potential odorant for a peripheral olfactory response in a salmonid, the background (control) water is first perfused over the rosette. After the control response is obtained, the odorant is delivered. This can be done at increasing strengths to determine the solution that elicits the maximum response. If neurotoxicants are tested, they are delivered following the initial testing of the odorant. Following the neurotoxicants, the control water is again delivered for thirty minutes, followed by retesting of the original odorant at the original strength to determine the amount of inhibition caused by the neurotoxicants. The difference in (negative) amplitude of the EOG is the amount of inhibition, expressed as a percentage of the original response.

There are several different types of non-overlapping olfactory receptor neurons (ORNs) that can detect several different classes of odor molecules. Chief among the classes of odorants that are known to affect specific ORNs are amino acids (Figure 2A & C), bile salts (Figure 2B), and prostaglandins. When testing potential neurotoxins, it has been found to be most effective to use multiple and dissimilar

odorants in order to determine whether the effect of a given neurotoxin is odor-specific or has a generalized effect on many different odorants and ORNs.

The Effects of Copper on Salmonid Peripheral Olfaction (EOG response)

Baldwin *et al*. (2003) examined the effects of copper on odor-evoked EOGs from the olfactory epithelium of coho salmon (*Oncorhynchus kisutch*) using three odorants. The odorants were L-serine, an amino acid, taurocholic acid (TCA), a bile salt, and a mixture of four amino acids (L-arginine, L-aspartic acid, L-leucine, and L-serine). Baseline EOG responses for all three odorants/odorant groups were obtained prior to the perfusion of the olfactory rosette with copper solutions. Nominal copper concentrations 1, 2, 5, 10 and 20 μg/L were added to the odorant solutions and used to perfuse the olfactory rosette. The dissolved copper concentrations of the perfusion solutions were measured. Measured concentrations were 87% ± 5% (mean ± SEM) of nominal plus background. Background copper concentration in the source water used in the experiments was 3.0 μg/L. Test results were reported as nominal copper concentrations (i.e. an increase over the background concentration of 3.0 μg/L). The potential ameliorating effects of calcium (water hardness) were also investigated in this study.

Copper reduced the amplitude of the EOGs for all three odorant groups. A 30-minute exposure to 10 μg/L copper reduced the EOG response of fish to L-serine, taurocholic acid (TCA), and an amino acid mixture by 57%, 67%, and 35%, respectively, of control (pre-copper exposure) EOG responses (Figure 3). Benchmark (threshold) concentrations (U.S. EPA 1995) were calculated from the nominal copper dose responses, which were normalized to the mean reduction in odor-evoked EOG responses of control animals. Benchmark concentrations of copper, based on a 25% reduction in response, were similar for all three odorants with estimates of 2.7, 2.3, and 3.0 μg/L, respectively, for L-serine, TCA, and the amino acid mixture. The benchmark values are nominal concentrations or an increase in copper over the background of 3.0 μg/L in the source water. These results indicate that

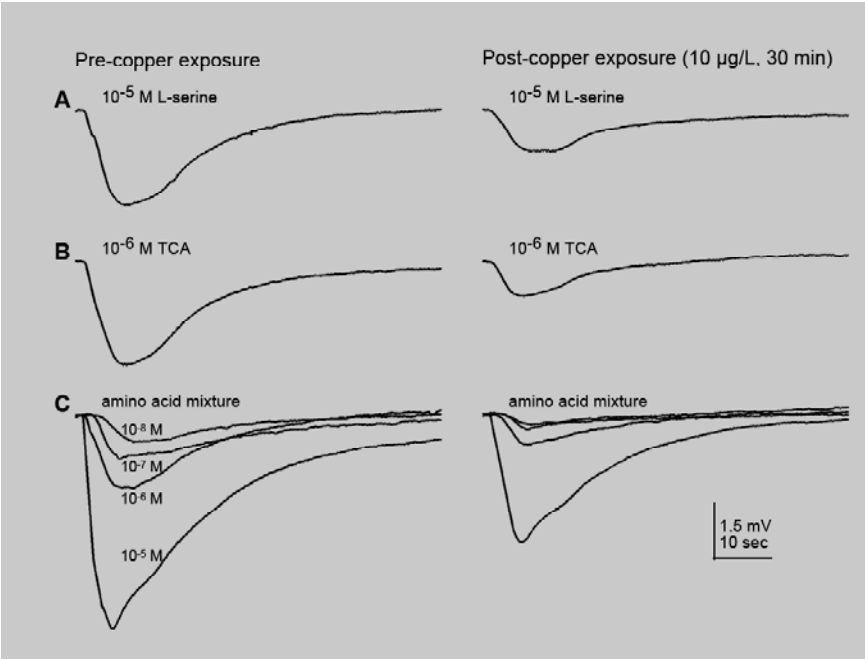


Figure 3. EOG responses (negative voltage transient) to odorants before (left) and after (right) a 10 μg/L copper exposure. Post-copper EOG responses were 57%, 67%, and 35% reduced (Figure 3, right) compared to control responses (Figure 3, left) to L-serine, TCA, and an amino acid mixture, respectively. Adapted from Baldwin *et al.* 2003.

copper may be a general neuroinhibitor, which affects different types of ORNs since it has been shown that microvillar ORNs respond to amino acids and ciliated ORNs respond to bile acids (e.g. TCA) in salmonids (Thommesen 1983).

In their study, Baldwin *et al*. (2003) also increased the hardness of the odorant/copper perfusion solutions from 20 to 120 and 240 mg/L ($CaCO_3$ equiv.), respectively, to measure the effect of water hardness on copper toxicity (inhibition) exhibited in the EOG response. The hardness of the source water used to perfuse the gills was not changed. There was no difference in inhibitory effects of 10 μg/L copper on ORNs based on the hardness values used in this study. These results are in sharp contrast to that of Bjerselius *et al.* (1993) who found that both high Ca^{2+} and high Mg^{2+} (e.g. high hardness) concentrations significantly reduced the immediate effects of Cu(II) exposure on the olfactory response. These researchers also found a significant correlation between Ca^{2+} concentration and the EOG response after recovery. Therefore, it is not known whether water hardness ameliorates the toxicity of copper to the olfactory epithelium, although it does ameliorate the acute toxicity of copper to fish (Miller and Mackay 1980) by competing with copper for metal binding sites on the gills and other tissues (Meyer *et al*. 1999).

Sandahl *et al.* (2004) examined the simultaneous effects of copper (and two other pesticides) on odor-evoked field potentials of the sensory epithelium (EOG) and olfactory forebrain (EEG) of coho salmon. The source (control) water used in these experiments had a hardness of 120 mg/L and contained 0.4 μg/L of total dissolved copper. Nominal concentrations of 0, 5, 10, and 20 μg/L copper were tested. Measured copper concentrations were 70-72% of nominal and the results were reported as nominal. Unlike the Baldwin *et al.* (2003) study, this study exposed fish to copper in aquaria rather than perfuse the copper over the olfactory rosette.

The 5 μg/L nominal copper concentration produced EOG and EEG responses that were not significantly different from control responses. The 10 μg/L copper concentration reduced the EOG and EEG responses by 47% and 40%, respectively, compared to control responses. Benchmark concentrations (U.S. EPA 1995) for 20% and 50% reductions in combined olfactory response (TCA-EOG, L-serine-EOG, TCA-EEG, and L-serine-EEG) were determined to be 4.4 and 11.1 μg/L, respectively. Seven-day exposures to 5, 10, and 20 μg/L copper (in aquaria) reduced the response to TCA and L-serine by approximately 20, 50, and 90%, respectively. An interesting and confounding aspect of the results was that the EOG response to TCA was reduced by approximately half following exposure to 5 μg/L copper, whereas the EEG response to TCA was not reduced at all. This surprising result, shown graphically in the research paper (Sandahl *et al*. 2004), is not discussed by the authors. This result (significant effect on EOG but none on the EEG) is of interest because paired EOG and EEG responses were obtained from the same fish. It should be noted that EOG and EEG responses to TCA following exposure to 10 μg/L copper were similar.

The Effects of Copper on Olfactory-mediated Behavioral in Salmonids

The effects of copper on salmonid behavior can be grouped into three categories. First, salmonids appear to exhibit avoidance behavior at very low copper levels (1.4-7 μg/L), perhaps even below any statistical or negative biological or neurophysiological effects level (e.g. Giattina *et al*. 1982). Second, copper in low to moderate amounts can inhibit salmonid olfactory (neurophysiological or behavioral) responses to certain ecologically important odors (Hansen *et al*. 1999a). Finally, salmonids may fail to avoid high (even lethal) concentrations of copper. This may be due to

impairment of the sensory mechanism or olfactory tissue, at least at very high (44 - 390 μg/L) copper concentrations (Hansen *et al*. 1999b; Giattina *et al*. 1982).

Typical laboratory avoidance experiments are conducted using a straight (Sprague 1964) or y-trough (Rehnberg and Schreck 1986) chamber that gives a fish the opportunity to choose control (reference) water or test water (control water + odorant and /or potential toxicant). Water flow and chemical concentration can be varied to produce either steep or shallow-gradient and either single or multiple exposures (Giattina *et al.* 1982). In a multiple exposure, the concentration of the odorant or neurotoxicant is increased over time.

Sprague (1964) conducted copper avoidance tests on Atlantic salmon (*Salmo salar*) parr using municipal water obtained from a soft water lake (hardness of 18 mg/L as $CaCO_3$). He derived an EC_{50} threshold avoidance value of 2.3 μg/L copper. The municipal (control) water contained a background level of 2 μg/L copper.

Rainbow trout may detect copper levels as low as 1.4-2.7 μg/L based on experiments where residence time in copper treated areas of the experimental chamber (along a copper gradient) began to decline at these levels. However, these levels were not statistically significantly different from the control (Giattina *et al*. 1982). This study also reported an estimated avoidance threshold for multiple exposures of 6.4 (95% confidence interval of 2.6-15.5) μg/L total copper for laboratory–conducted avoidance tests. The single exposure threshold value reported for copper was 7.3 μg/L (Giattina *et al* 1982).

One of the most noteworthy studies on copper avoidance by salmonids compared the sensitivities of Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) to copper using copper-spiked simulated creek water (Hansen *et al.* 1999a). Well water was diluted with de-ionized water to obtain a hardness, alkalinity, and pH of 25 mg/L, 28 mg/L, and 7.5, respectively, to simulate a mine-affected stream (Panther Creek) in Idaho. Chinook salmon avoided 0.8 and 2.8 - 22.5 μg/L copper but did not avoid 1.6 μg/L copper. The failure to obtain a significant avoidance at the 1.6 μg/L copper concentration was attributed to a small sample size (n=10). The authors argued that their study showed avoidance at such low levels (i.e. 0.8 μg/L) due to a larger sample size (n=20) than past researchers (study sample sizes were 10 or 20). Following acclimation to 2 μg/L copper for 25-30 days, Chinook salmon failed to avoid any of the copper concentrations tested (3.4 - 21 μg/L).

In contrast to Chinook salmon, rainbow trout avoided 1.6 – 88 μg/L (Hansen *et al*. 1999a). The maximum copper avoidance for rainbow trout was between 5 and 50 μg/L, where fish averaged less than 10% of their time in the contaminated end of the chamber, whereas the maximum copper avoidance for Chinook salmon was at 6 μg/L. Following acclimation to 2 μg/L copper for 25-30 days, rainbow trout preferred clean water and avoided all copper concentrations greater than the reference control of 1.6 μg/L ($p<0.05$). Thus, acclimation to copper (2 μg/L) did not affect rainbow trout responses to copper. However, acclimation to copper did affect all subsequent responses of Chinook salmon to all copper concentrations (i.e. they did not avoid any subsequent copper concentration tested; $p>0.05$).

The enigmatic attraction of salmonids to copper at high concentrations was reported by Giattina *et al*. (1982). They found that rainbow trout (*Oncorhynchus mykiss*) were attracted to total copper

concentrations of 330-390 μg/L during shallow-gradient tests. Similar to other olfactory experiments (e.g. Rehnberg & Schreck 1986: Baldwin *et al*. 2003; Hansen et. al. 1999a & b), the water used in these experiments was soft, having a hardness of 28.4 mg/L and an alkalinity of 25.2 mg/L (as $CaCO_3$). The significance of this is discussed in a later section. The work of Hansen *et al*. (1999a) appeared to confirm this finding. They found that rainbow trout failed to avoid 180 and 360 μg/L copper and that Chinook salmon did not avoid 44 - 340 μg/L copper. Failure to avoid high copper concentrations may be due to olfactory cell destruction by high copper concentrations, but other potential inhibitory mechanisms (Cu binding to cell membrane ligands) may also be involved (Hansen *et al*. 1999b).

In contrast to the results of Giattina *et al.* (1982) and Hansen *et al* (1999a), Rehnberg & Schreck (1986) found that a different salmonid, coho salmon (Oncorhynchus kisutch), did avoid high copper levels of 566 μg/L.

The main issue that provided the impetus for this review was the observation that copper interfered with salmonid olfactory perception of ecologically important odors. As mentioned previously, perception of amino acids and bile salts in their environment may be critical to a salmonid's ability to avoid danger, migrate to natal streams and spawn. In addition to the neurophysiological evidence (EOG & EEG) already presented, two neurobehavioral studies were reviewed, which demonstrated the effects of copper on salmonid behavior. A third study, which linked neurophysiological and neurobehavioral results, is discussed in the following section.

Lorz and McPherson (1976) exposed 10-18 month old coho salmon (O. *kisutch*) to nominal copper concentrations of 0, 5, 10, 20, and 30 μg/L (as copper chloride) under flow-through conditions in 1000 L tanks for 24 to 4128 hours. Nominal copper concentrations were added to well water having 84-99 mg/L (as CaCO3) hardness and less than 2 μg/L copper. Following copper exposures, the salmon were released to a natural creek during the normal downstream migration period (April to June 1975). The number of (control and copper treated) tagged fish entering a trap 6.4 km downstream over a period of 29 days was used to determine the effect of copper on downstream migration. Fish exposed to all copper concentrations (5, 10, 20 and 30 μg/L) for 3960 hours (165 days) had reduced downstream migration compared to control fish. Fish exposed to 30 μg/L copper for 1632 hours had an approximate reduction in downstream migration of 30% compared to control fish. An exposure to 30 μg/L copper for as little as 72 hours caused a substantial reduction in migration (52% movement vs. 93% movement in control fish). Thus, sublethal concentrations of copper, applied beforehand, can affect downstream migration. The concurrent LC_{50} for juvenile coho salmon determined in the same laboratory was 60-74 μg/L copper (Lorz and McPherson 1976). However, it should be noted that although the 5 μg/L copper exposure reduced downstream migration by about 16-18%, it had no effect on gill ATPase activity or survival in seawater (two other aspects of the study).

Rehnberg & Schreck (1986) found that avoidance of 10^{-8} M L-serine (a known alarm chemical) by coho salmon was inhibited by copper concentrations of $10^{-7.2}$, $10^{-6.05}$, and $10^{-5.05}$ M (4.01, 56.6, and 566 μg/L, respectively). Interestingly, they found through binding assays that the inhibitory aspects of Cu^{2+}, seen in the behavioral assays, could not be explained by interactions at the serine receptor. In contrast, Hg^{2+} clearly inhibited serine binding to the olfactory receptor. In addition, they found that copper does not bind with serine in significant amounts at copper concentrations of 10^{-6} to 10^{-7} M. This ruled out any speculation that the inhibition caused by copper could be explained by the formation of non-stimulatory, metal-serine complexes. Copper is about 50% bound to serine at a

concentration of about 10^{-5} M (635 μg/L), which is well beyond the lethal limit for any salmonid (U.S. EPA 1985a & 1996). The mechanism by which copper inhibited detection of L-serine could not be determined in this study.

Other Effects of Copper on salmonid olfaction

Hansen *et al*. (1999b) linked neurophysiological and histological effects of copper to the behavioral differences in copper sensitivity of rainbow trout and Chinook salmon found in a companion study (Hansen *et al*. 1999a). The two studies used the same water quality conditions and copper concentrations. Rainbow trout exposed to 25, 50 and 100 μg/L copper concentrations had EEG responses to L-serine that were 50-65% of control responses. Trout exposed to 200 and 300 μg/L copper had EEG responses that declined over a 60-minute exposure period until the EEG response was virtually eliminated. Chinook salmon EEG responses to 25 μg/L copper were reduced by about 50% (Hansen *et al.* 1999b). All higher concentrations of copper caused the EEG responses of Chinook salmon to decline over a 60-minute exposure until they were indistinguishable from spontaneous EEG activity.

Spontaneous EEG responses of rainbow trout to copper were significantly greater than those for Chinook salmon and these responses were not dependent on copper concentration (Hansen *et al*. 1999b). Following a one-hour exposure, the number of olfactory receptors was significantly reduced in Chinook salmon (≥ 50 μg/L copper exposure) and in rainbow trout (≥ 200 μg/L copper exposure). The number of olfactory receptor cells was reduced in both species when exposed to a copper concentration of 25 μg/L for four hours. This study concluded that copper stimulates the olfactory system, directly or indirectly, as evidenced by a spontaneous increase in EEG amplitude following copper exposure. It is this stimulation of the olfactory system that may lead to copper avoidance (Hansen *et al*. 1999b).

These researchers found a dose-response relationship of copper concentration to the EEG response to L-serine following copper exposure. Exposing fish olfactory epithelium to greater copper concentrations resulted in a decline in magnitude and rate of the initial response to L-serine and a slower rate of recovery following copper exposure. The study suggests that two different mechanisms might account for increasingly more severe results observed under increased copper concentrations. They argued that low copper concentrations (25 μg/L), which reduced EEG activity by 50%, may impair ion pumps or block ion channels, causing interference with the signal transduction, as has been shown in channel catfish (Restrepo *et al*. 1990). Higher copper concentrations (50 μg/L) initially reduce the EEG in the same way as lower copper concentrations. However, irreversible effects on the EEG, observed after prolonged exposures to higher copper concentrations, appear to be caused by reductions of intact olfactory receptors on the epithelial surface of the olfactory rosette of tested fish (Hansen *et al*. 1999b). Using transmission electron microscopy (TEM), they observed ruptured receptor membranes, loss of cilia and microvilli, and swollen mitochondria in tissue taken from the olfactory rosette. This observation appeared to confirm the reason for the total loss of EEG response to L-serine at high copper exposures.

Hara *et al*. (1976) found that 50 μg/L copper (as $CuSO_4$), administered for two hours, depressed the olfactory bulbar response (EEG) by about 50% of the original level (control response). Full recovery of the bulbar response to L-serine was not achieved even after rinsing for two hours with dechlorinated water. The threshold concentration of copper that caused minimal depression of the

bulbar response was 8 μg/L, added to the lab water. The lab water contained 20 μg/L of background copper.

Comparison of EPA Copper Criteria to Olfactory Effects Levels

The 1984 ambient freshwater quality criteria for copper (U.S. EPA 1985a) were updated in 1995 (U.S. EPA 1996) and promulgated in the California Toxics Rule (U.S. EPA 2000). The acute (Criterion Maximum Concentration or CMC) and chronic (Criterion Continuous Concentration or CCC) are hardness dependent since the toxicity of copper to freshwater aquatic organisms is dependent on the amount of calcium (especially) and magnesium in the water (U.S. EPA 1985a). The freshwater CMC and CCC are 13 and 9.0 μg/L, respectively, based on a hardness of 100 mg/L. However, these reported (table) values (e.g. U.S. EPA 2000), standardized to 100 mg/L hardness, can be deceiving. For example, an acute value of 2.8 μg/L for rainbow trout was reported in the EPA 1995 Updates (U.S. EPA 1996). This acute value was determined from a test in which the water hardness was 9.2 mg/L. At first glance, the CMC of 13 μg/L would appear to be under protective of the 2.8 μg/L copper result reported for rainbow trout. However, the copper CMC, when re-computed for a hardness of 9.2 mg/L is actually 1.4 μg/L, which would be protective of the acute value of 2.8 μg/L reported for rainbow trout. Thus, the criteria for waters of differing hardness values cannot be directly estimated by looking only at the table's criterion based on a hardness of 100 mg/L, especially if reported effects results are for soft waters.

One of the observations presented by Baldwin *et al.* (2003) was that Pacific salmon populations were in decline and that salmonids, which have a highly developed sense of smell, may be subject to sub-lethal neurotoxicity from copper (and other potential toxicants) in storm water runoff entering streams inhabited by salmon. They reported that copper concentrations typical of those that have been measured in surface waters from urban and agricultural watersheds had significant or at least threshold effects on the salmonid peripheral nervous system, as measured by EOG responses. They questioned whether the current aquatic life criteria for copper (CMC of 13 and CCC of 9.0 μg/L based on a hardness of 100 mg/L) was protective of the loss of olfactory function seen in the EOG at lower copper concentrations.

The species-specific olfactory effects levels reported in the studies reviewed were compared to the current aquatic life criteria based on the hardness of their specific test waters (Appendix 1). Twenty-five of the most sensitive endpoints (worst-case scenario) from nine studies were evaluated with respect to the EPA criteria (Appendix 1). Five of the 25 reported endpoints were more sensitive (lower) than the corresponding hardness-based EPA criteria. Of these five, one was from a test in which the hardness had been artificially raised to 240 mg/L using $CaCl_2$ (Baldwin *et al*. 2003), two were from a study in which well water had been diluted with de-ionized water (Hansen *et al.* 1999a), one involved a 165-day copper exposure and had an endpoint effect which was less than 20% different from the controls (Lorz & McPherson 1976), and one had a calculated benchmark reduction in combined EOG and EEG responses to two odorants of 20% (Sandahl *et al.* 2004). These five endpoints and the corresponding EPA-based CCCs (the most sensitive criterion) are shaded in Appendix 1.

Water hardness ameliorates the toxicity of copper to aquatic organisms including Ceriodaphnia dubia (Naddy *et al.* 2003), *Daphnia magna* (De Schamphelaere and Janssen 2002), and rainbow trout (Taylor *et al.* 2000). These are representative species of the 1st, 2nd, and 12th most sensitive genera out of the 43 genera in the 1995 EPA database (U.S. EPA 1996). The ameliorative effects of calcium

appear to be greater than those of magnesium for fish (Welsh *et al.* 2000), although for invertebrates magnesium may have similar protective effects (Gensemer et al. 2001). The hardness-based copper criteria values (U.S. EPA 1996) illustrated in Appendix 1 do not distinguish between calcium and magnesium hardness. However, the recent draft update of the copper criteria does attempt to distinguish the contribution of calcium and magnesium to the reported hardness in toxicity studies (U.S. EPA 2003).

The EOG has been used to determine the effect of calcium and magnesium (i.e. hardness) on the inhibitory effects of copper on salmonid peripheral olfaction with mixed results. Baldwin *et al*. (2003) reported that adding calcium (see Appendix 1) did not ameliorate the inhibitory effects of a 30-minute exposure to 10 μg/L copper, on olfactory responses of coho salmon to L-serine. Bjerselius *et al.* (1993) reported that the suppression of the EOG response in Atlantic salmon to L-alanine by 635 μg/L copper was significantly less in solutions with high calcium (4000 μM or 400 mg/L as $CaCO_3$) or high magnesium (3600 μM or 360 mg/L as $CaCO_3$). Also, there was a significant correlation between the Ca^{2+} concentration and the EOG response after recovery. In contrast, 3600 μM Mg^{2+} had no effect on recovery. These researchers (Bjerselius *et al.* 1993) hypothesized that high Mg^{2+} concentrations reduced the immediate effects of Cu(II) exposure by increasing the ionic strength of the solution, thereby lowering Cu^{2+} activity.

Based on the work of Baldwin *et al.* (2003), the role of calcium in ameliorating toxicity is not likely the same for olfactory sensory cells as it is for fish gills (biotic ligand model). The role of calcium in the fish gill model is that it competes with copper for binding sites, decreasing the toxic effect of copper (Meyer *et al.* 1999). If calcium exhibited competitive binding at the surface of olfactory sensory cells, Baldwin *et al.* (2003) would have observed decreasing neurotoxicity (as shown in the EOG response) due to copper with increasing calcium concentrations of the perfusion water. However, that was not the case. Nonetheless, calcium may play an important role in olfactory transduction. For example, in channel catfish (*Ictalurus punctatus*), stimulation of olfactory neurons elicited an influx of calcium that lead to an increase in intracellular calcium levels (Restrepo *et al.* 1990).

In 2003, the EPA released a draft update of the ambient water quality criteria for copper (U.S. EPA 2003). The proposed freshwater criteria are based on the biotic ligand model or BLM (DiToro *et al.* 2001; Santore *et al*. 2001; Heijerick *et al*. 2002). The two main components of the BLM are competitive ions, such as calcium (already discussed) and metal speciation. It is the bioavailable species (principally the ionic form) of metal that is toxic to aquatic organisms (U.S. EPA 2003). This is true for metal exposure to the gill (or other biotic ligand) as well as to the olfactory epithelium of salmonids, as seen in EOG responses. For example, Bjerselius *et al*. (1993) found a significant correlation between the free Cu^{2+} activity and the EOG response of Atlantic salmon after four minutes of copper exposure. Winberg *et al*. (1992) also used Atlantic salmon to demonstrate that the toxic effect of copper is caused mainly by the dissolved Cu^{2+} ion. The different EOG profiles (initial peak and sustained components of the EOG) obtained by these researchers in response to Cu^{2+} ion concentrations indicated that this ion affects the olfactory transduction mechanism at different stages of transduction.

It is impossible to directly apply the BLM model to the nine olfactory studies reviewed in this paper (Appendix 1). In general, these studies did not report all of the parameters required to run the model [temperature, pH, dissolved organic carbon or DOC, major cations (Ca, Mg, Na, K), major anions (SO_4, Cl), alkalinity and sulfide]. The most important parameter not reported was organic carbon.

Of the nine studies (Appendix 1), none were run in natural water. The use of soft to moderately hard (20-120 mg/L hardness as $CaCO_3$) municipal or well water in all the tests (one test artificially raised hardness to 240 mg/L) likely meant that a larger percentage of the added copper (nominal or measured dissolved or total) was ionic Cu^{2+} (with some inorganically-bound copper) than would be typical in a natural water, and therefore the copper in the exposure water was more bioavailable than it would be in a natural water. Bjerselius *et al.* (1993) calculated that ionic copper made up 94% of the total copper concentration in their studies (Appendix 1). In addition, municipal and well water are not likely to have a significant amount of total or dissolved organic material (TOC/DOC). Giattina *et al*. (1982) reported that copper analytical procedures were modified (reduced ashing time for atomic absorption spectrophotometry) due to the "low organic content of the test water."

The failure to consider the organic content of salmonid home streams in conducting olfactory tests was especially evident in one of the nine studies reviewed. Hansen *et al.* (1999a) conducted behavioral avoidance experiments on Chinook salmon and rainbow trout "under water quality conditions and metals concentrations that simulated a mine-affected stream, Panther Creek in Idaho, USA, where both species once resided." However, the simulation consisted of diluting well water with de-ionized water. The pH of the water was adjusted using sodium hydroxide or sulfuric acid. Obviously, the organic content of Panther Creek was not simulated (nor was it reported). While the creek may have had low DOC, it is not likely that it was lower than well water, which had a TOC of 0.3 – 0.4 mg/L (J. Hansen, personal communication). The further dilution of the well water with pure (de-ionized) water would have decreased the DOC of the well water even further, rather than have "simulated" the organic content of the creek water. This manipulation of test water (dilution with pure water) would have increased the bioavailability of copper in the test water, and was likely responsible for the lowest copper effects levels found in this review (Appendix 1).

As an illustration of the importance of DOC in the test conditions, the BLM can be used to extrapolate the copper effect levels from the conditions in a test to conditions that are more representative of natural waters. An extrapolation was performed using the results from Hansen *et al.* (1999a), the approximate water quality for that study reported in Hansen *et al*. (2002), and assuming a dilution factor of approximately 8.6 (hardness of 217 mg/L diluted to 25.3 mg/L, as $CaCO_3$). Using this dilution factor, the test water (Hansen *et al.* 1999a) contained approximately 0.03 mg C /L as DOC. The BLM predicted that the lowest effect level observed by Hansen *et al*. (1999a) of 0.8 μg/L would have been approximately 34 μg/L in test water with a DOC of 2 mg C/L. Clearly there is likely to be a significant effect due to even low amounts of organic carbon.

One final question remains concerning the EPA acute and chronic criteria for copper and the olfactory effects reported in this review, namely, are the study data presented in this review (Appendix 1) considered suitable (i.e. of high enough quality) for use in criteria development by the EPA? That question is difficult to answer for results reported since 1999 because the EPA was either not aware of them (e.g. Hansen *et al.* 1999a & b) or the studies occurred or were reported after the latest EPA criteria update (Baldwin *et al*. 2003, Sandahl *et al*. 2004). For study results reported prior to 1999, the EPA used none of the five earlier studies (Appendix 1) for criteria development. Four of these five studies, Lorz & McPherson (1976), Hara *et al*. (1976), Giattina *et al.* (1982), and Rehnberg & Schreck (1986) were listed under unused data in either the 1984 (U.S. EPA 1985a) or the 2003 (U.S. EPA 2003) updates. The fifth study by Sprague (1964) was not on either the used or unused lists, although a companion study conducted in the same year (1964) was listed under unused data. Data that are placed on the unused data list are from studies that the EPA does not consider "suitable" for criteria development (U.S. EPA 2003). The most prominent example of the exclusion

of olfactory data by the EPA was the Lorz & McPherson (1976) study data. The 1984 copper criteria development report (U.S. EPA 1985a) used three acute endpoints for coho salmon (EC_{50} values of 60, 70 & 74 μg/L copper) from the Lorz & McPherson study for their criteria determination effort. But the EPA report rejected results from the same study (Appendix 1) showing inhibition of downstream migration when fish were pre-exposed to copper concentrations of 5 and 10 μg/L.

Ecological Relevance

The toxicity of copper to aquatic organisms is due mainly to the cupric ion (Cu^{2+}), the bioavailable fraction of the metal (see review in U.S. EPA 2003, p.2). The cupric ion is also responsible for inhibitory effects on the peripheral olfactory sensory tissues of salmonids (Bjerselius *et al*. 1993; Winberg *et al*. 1992). Winberg *et al*. (1992) observed that the form and amplitudes of Atlantic salmon EOG responses to L-alanine changed "drastically" with increasing concentrations of Cu^{2+}, even though the total amount of copper in the irrigation solutions remained the same. Certain water quality parameters (listed above) affect the speciation of copper and consequently its bioavailability. These parameters include pH, alkalinity, organic carbon, and suspended solids. Each of these, when increased, would lower the concentration of Cu^{2+}, thereby decreasing the toxicity of the sample. National criteria are derived in laboratory waters, which are low in suspended solids and organic carbon. They are derived in a manner intended to protect all or almost all bodies of water, and therefore are likely to be at least somewhat overprotective for a majority of waterbodies (U.S. EPA 1985b). The water used in the copper/olfactory studies described in this review (well water and de-chlorinated municipal water) would likely meet the requirements of U.S. EPA (1985b) for low suspended solids and organic carbon, and therefore test endpoints derived in these waters may be similarly overprotective of many natural surface waters.

The ecological relevance of salmonid olfactory studies conducted in soft water of low ionic strength and low organic carbon is difficult to determine since the bioavailable fraction of copper in these studies may be much higher than for natural surface waters. Some of the reported effects levels for salmonid olfactory inhibition are at or below the current applicable EPA hardness-based criteria (Appendix 1). However, there may be site-specific factors in surface waters, such as dissolved organic carbon and suspended particles, which ameliorate the impact of copper. None of the control waters used in the nine studies reported in Appendix 1 would likely have had appreciable organic carbon. One study reported total organic carbon (TOC) of <2 mg/L (Rehnberg & Schreck 1986). Another study reported that the organic carbon content of the test water was low but did not report the amount (Giattina *et al*. 1982). A third indicated that the only organic ligand present in the test water would have come from the added odorant, L-alanine (Winberg *et al*. 1992). As mentioned, the calculated DOC of the diluted test water used in the Hansen et al. (1999a) study was approximately 0.03 mg/L.

It could probably be assumed that the TOC of the test waters for studies reported in Appendix 1 were all <2 mg/L. In contrast, the estimated TOC for California streams is 9.33 mg/L (reported in U.S. EPA 2003 – the reference did not state whether this was a mean or median value). The effect of low TOC on these olfactory test results cannot be directly determined, with the exception of the Hansen *et al.*(1999a) study previously discussed. In order to better estimate the ecological relevance of olfactory test results, whether based on neurophysiological (i.e. EOG or EEG) or neurobehavioral (i.e. "predator" odor avoidance) endpoints, future salmonid olfactory studies would be well served to measure and vary the amount of organic carbon in the test water. Applying the BLM model to studies of the neurotoxicity of copper to salmonids is a logical next-step in establishing the ecological relevance of such studies. While copper may enter urban streams through stormwater runoff, humic

acids and other organic ligands and suspended solids are likely to concurrently enter the streams, decreasing the amount of bioavailable copper present.

In order to use olfactory impairment studies for criteria development (e.g. copper), or to extrapolate laboratory study results to the natural environment of streams, an important methodological consideration is to ensure that the control water is not contaminated with the experimental variable (e.g. copper). Some of the studies reviewed had control waters with background copper concentrations equal to or greater than the effect seen (Baldwin *et al.* 2003; Hara *et al*. 1976; Sprague 1964). Baldwin *et al*. (2003) reported 25% benchmark reductions in EOG responses of coho salmon to three odorant/odorant combinations of 2.3, 2.7, and 3.0 μg/L. However, the background copper concentration of the control water was 3.0 μg/L. Since the amplitude of the control water EOGs was empirically pre-set by varying the concentration of the odorant and the odorant pulse interval prior to testing, does this mean that the copper in the control water had no effect on the EOG? Do coho salmon in natural streams exhibit a response similar to that of laboratory fish exposed to the same odorant concentration? Evans & Hara (1985) reported that the "EOG response to amino acids increased nearly exponentially with concentration and no saturation was reached." Based on that result, it cannot be said with certainty that the in-stream effect of the further addition of 2.3 – 3.0 μg/L copper would have caused the same (25%) reduction in EOG response.

All of the studies reviewed in this paper focus primarily on the effect of copper in freshwater. Many vital activities of salmonids occur in freshwater, including spawning, migration, kin recognition, and predator avoidance, especially by immature animals. Some limitations on the extrapolation of the laboratory results to natural creeks have been discussed. Notwithstanding these limitations, elevated free ionic copper levels in creeks that are known to be soft, poorly buffered and low in organic content, may pose risks to salmon and trout populations that inhabit them.

Important future work with neurotoxicants (e.g. copper) would be to link salmonid neurophysiological results to ecologically significant behavioral results (Baldwin *et al.* 2003; Sandahl *et al*. 2004). While electrophysiological and histological results have been compared in the same study (Hara *et al.* 1976), this has not been done with behavioral results (Baldwin *et al*. 2003). "Evaluating the effects of the partial loss of sensory capacity on behavioral function is considerably more complex [than evaluating total loss], and this is an important area for future research" (Baldwin *et al*. 2003).

A search of the available literature did not reveal any studies on the effects of copper on salmonid olfaction in seawater. However, much of the above discussion may be extrapolated to seawater, with the understanding that the ionic concentration and composition of seawater is much greater than that of freshwater. Since increasing ionic concentration decreases cupric ion activity (Bjerselius *et al.* 1993), one would expect that the fraction of total copper that is bioavailable in seawater to be much less than in freshwater. One example of this is the substantial difference in the Species Mean Acute Values (U.S. EPA 2000) for coho salmon (*Oncorhynchus kisutch*) in freshwater (15.98 μg/L) and in seawater (546.3 μg/L).

Moreover, the Water-Effect Ratio studies completed in North and South San Francisco Bay (Bay) indicate that bioavailability of copper in the Bay is reduced compared to that in clean laboratory seawater. The established and proposed site-specific dissolved copper water quality objectives (SSOs) for the Bay are 6.0 μg/L (for Bay Regions 1-3) and 6.9 μg/L (for Bay Regions 4 & 5). It is difficult to directly compare the results of the reviewed studies conducted in freshwater to the Bay

SSOs developed for estuarine waters. All study waters were very soft to moderately hard and from wells or municipal sources that would likely have low organic carbon content. For the most part, the reviewed studies did not consider or vary the amount of organic carbon in their test waters. However, one way to evaluate the protectiveness of the proposed SSOs can be demonstrated as follows.

The most sensitive endpoint identified in the literature review (Appendix 1) was 0.8 μg/L ($10^{-7.9}$ M) for Chinook salmon (Hansen *et al*. 1999a). This test was run in well water diluted with de-ionized water, to a hardness and alkalinity of approximately 25 mg/L (as $CaCO_3$). Extensive water quality data for this test was obtained from the original author (J.A. Hansen, personal communication; Hansen *et al*. 2002), which allowed use of the Biotic Ligand Model to calculate the fraction of free ionic copper in the test water. The BLM estimated that under the described test conditions the free copper ion concentration would be 4.4 X 10^{-10} M, or three orders of magnitude greater than the current free hydrated Cu^{2+} ion concentrations of 10^{-13} M measured as several locations throughout the Bay (Buck & Bruland 2005). In other words, Cu^{2+} concentrations in the Bay would have to increase more than 1,000 times before reaching levels that may inhibit olfaction in Chinook salmon, the species identified in the present literature review whose olfactory function was found to be the most sensitive to copper.

Conclusion

The "Impairment Assessment Report for Copper and Nickel in Lower South San Francisco Bay" (Tetra Tech, Inc., *et al*. 2000) and the "North of Dumbarton Bridge Copper and Nickel Conceptual Model and Impairment Assessment Report" (EOA, Inc. & Larry Walker Associates 2004) concluded that impairment of beneficial uses in the Bay due to copper and nickel is unlikely. The current review addresses the protection of salmonid species in the Bay due to potential olfactory impairment by copper. The available information on olfactory impairment by copper is limited to laboratory tests in fresh waters that are low in hardness and organic carbon, and is therefore difficult to extrapolate to the estuarine and marine conditions existing throughout most of the Bay. There has been no measurable change in Lower South Bay ambient dissolved copper concentrations since adoption of the 6.9 μg/L SSO in 2002. Similarly, there is no expectation that there would be ambient increases North of Dumbarton Bridge solely due to adoption of the proposed 6.9 and 6.0 μg/L SSOs. Similar copper monitoring and control measures will be in place to ensure this as part of the concurrently adopted Copper Management Strategy.

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Glossary

α-Amino Acid – An alpha-amino acid has the amine group on the alpha carbon, that is, the carbon adjacent to the carboxylic acid group.

Acetylcholine – A white crystalline compound that transmits nerve impulses across intercellular gaps.

Acetylcholinesterase (AChE) – An enzyme that hydrolyzes acetylcholine to form acetic acid and choline.

Acidification – The process of making acidic or lowering the pH of a substance (e.g., the acidification of streams refers to the influence of acid rain or the decomposition of organic material which can lower stream pH).

Ameliorate – To make or become better; improve.

Anosmic – Loss of the sense of smell.

ATPase – Adenosine triphosphatase - An enzyme that catalyzes the hydrolysis of ATP (adenosine triphosphate) to ADP (adenosine diphosphate), releasing energy that is used in the cell.
Bile Acids – Any of the liver-generated steroid acids that appear in the bile as sodium salts.

Biotic Ligand Model (BLM) – The BLM is a gill (or other biotic ligand) model of toxicity which incorporates two basic factors which affect toxicity: 1) metal speciation; and 2) competitive ion binding. The model predicts the free ionic (bioavailable) concentration of the metal and the amelioration of toxicity (metal binding at the gill or biotic ligand) due to competition from other ions (chiefly calcium, but also magnesium, sodium, and potassium).

Cauterization – A burning or searing with a caustic agent or a very hot or cold instrument in order to destroy tissue.

Charr (also Char) – A fish of the genus *Salvelinus* that is related to trout (Family: Salmonidae). This common name refers especially to the widely-distributed *Salvelinus alpinus*.

Chemosensory Recognition – Relating to the sensory reception and recognition of a chemical stimulus.

Cilia – Microscopic hair-like processes extending from a cell surface. Olfactory receptor neurons (ORNs) are nerve cells that may have microvillar or ciliated projections.

Conspecifics – Members of the same species.

Criterion Continuous Concentration (CCC) – The highest concentration of a pollutant which should not unacceptably affect aquatic organisms if the 4-day average of this concentration is not exceeded more than once every three years.

Criterion Maximum Concentration (CMC) – The highest concentration of a pollutant which should not unacceptably affect the short-term survival of aquatic organisms if the one-hour average of this concentration in not exceeded more than once every three years.

D-Isomers – Refers to the arrangement of atoms of a compound in space (e.g., D-glyceraldehyde has the OH group on the right and is a mirror image of the L-isomer).

Electroencephalogram (EEG) – A recording of the electrical activity of the brain, forebrain, or olfactory bulb in fish.

Electro-Olfactogram (EOG) – A recording of an electronegative wave of potential occurring on the surface of the olfactory epithelium in response to stimulation by an odor.

Expressible Milt – Milt is fish sperm which includes the seminal fluid. "Expressible" indicates that the fish has been sexually primed, that the sperm are mature and activated, and that the fish is ready to spawn.

Gonadal Steroid – sex hormone.

Hydrolyze – To decompose a chemical compound by reaction with water (i.e., to undergo hydrolysis).

Immunoreactive – Pertaining to an immunologic reaction, especially in vitro between antigen and antibody.

L-Isomers – Refers to the arrangement of atoms of a compound in space (e.g., L-glyceraldehyde has the OH group on the right and is a mirror image of the D-isomer). L-isomers of amino acids are contained in protein and therefore tend to be more "active" in stimulating olfactory responses in fish.

L-Serine – An amino acid ($C_3H_7NO_3$) that is a common component of many proteins and which stimulates an avoidance response in salmonids. The L-isomer is more "active" in producing an olfactory response than the D-isomer.

Metal Speciation – The chemical forms or fractions that comprise the total metal concentration. In general they are the ionic, inorganically complexed, organically complexed, and particulate bound metal complexes. Metals are generally measured as total or dissolved, but ionic concentrations (the most bioavailable fraction of the metal) can also be measured or estimated from various water quality parameters.

Microvilli – Microscopic projections from the surface of a cell. Nerve cells that may have microvillar or ciliated projections include Olfactory receptor neurons (ORNs).

Nares: The plural of naris. Naris is an opening in the vertebrate nasal cavity (nose). Fish have two (nares).

Natal Stream – A stream where fish are born.

Neurobehavioral – Pertaining to the study of the effects of the nervous system on behavior.

Neurophysiological – Pertaining to the physiology of the nervous system.

Neurotoxicant (also neurotoxin) or Neuroinhibitor – A toxin or inhibitor that acts specifically on nerve cells (neurons), usually by interacting with membrane proteins and ion channels.

Neurotransmitter – A chemical substance that transmits nerve impulses across a synapse (e.g., acetylcholine or dopamine).

Odorant: A substance that stimulates the sense of smell. Fish odorants include amino acids (especially the L-isomers), serine, glycine, alanine, asparagine, cysteine, histidine, methionine, norvaline, norleucine, and glutamine.

Olfactory Bulb – One of two enlargements at the terminus of the olfactory nerve at the base of the brain just above the nasal cavities.

Olfactory Epithelium – The membranous tissue of the olfactory rosette which contains the olfactory receptor neurons (ORNs) in salmonids. A single ORN is made up of cilia or microvilli at the surface,

a dendrite leading to the soma and an axon at the base of the cell. The axons of many ORNs combine to produce the olfactory nerve.

Olfactory Receptor Neurons (ORNs) – Neurons in the olfactory epithelium with proteins that bind and detect odorants. A neuron is a nerve cell that consists of a nucleated portion and cytoplasmic extensions, the cell body or soma, dendrites, and axons.

Olfactory Rosettes – A pair of odorant receptors located within the nares of the fish. The rosettes are so named because they are round and contain several folds of lamellae covered with sensory epithelium.

Organic Material (DOC) – Dissolved Organic carbon is a measure of organic matter (carbon) present in a sample that has been passed through a 0.45 □m pore-size filter.

Parr – A salmon during the first 1.5 - 2 years of its life when it lives in freshwater.

Perfuse – To pour or diffuse over.

Peripheral Olfaction – The sensory response to odors exhibited in the olfactory rosette epithelium (organ) where the sensory nerves end.

$PGF_{1\alpha}$ & $PGF_{2\alpha}$, – F-type prostaglandins. Sex pheromones. See Prostaglandins.

Pheromone, Alarm pheromone – A chemical substance secreted by an animal that influences specific patterns of behavior (for example, alarm) by other members of the same species.

Plasma Hormone – Blood hormone level.

Positive Rheotaxis - Swimming upstream when the odor is present, and backtracking downstream when the home odor is absent.

Preovulatory – Pertaining to a sexually immature female fish which has not released eggs from its ovary.

Prostaglandins – A group of compounds derived from unsaturated 20-carbon fatty acids. They are extremely potent mediators of a diverse group of physiological processes.

Salmonids – Soft-finned fishes of the family Salmonidae including trout, salmon, and charr.

Serine Receptor – An olfactory receptor neuron (ORN) that binds with serine, an amino acid and common salmonid avoidance odorant.

Smolt – A young salmon at the stage at which it migrates from freshwater to the sea.

Smoltification – The processes whereby salmon parr become smolts. A suite of physiological, morphological, biochemical and behavioral changes, including development of the silvery color of adults and a tolerance for seawater, that take place in salmonid parr as they prepare to migrate downstream and enter the sea.

Transduction – The transfer of one type of input to another type of output (e.g., the transfer of the recognition of an odor molecule by a receptor cell into an electrical impulse stimulating the olfactory bulb or forebrain).

Transmission Electron Microscopy (TEM) – An imaging technique whereby a beam of electrons is focused onto a specimen causing an enlarged version to appear on a fluorescent screen or layer of photographic film.

Appendix 1. Comparison of EPA hardness-based copper criteria with endpoints of studies reviewed (shaded results indicate effects that are potentially more sensitive than the hardness-based CCC). AS - Atlantic salmon (Salmo salar); CCC - Criterion Continuous Concentration; CMC - Criterion Maximum Concentration; CS - coho salmon (Oncorhynchus kisutch); ChS - Chinook salmon (O. tshawytscha); D - Dechlorinated; DW - Deionized Water; EOG - Electro-olfactogram; F - Filtered; M - Municipal water source; RT - Rainbow trout (O. mykiss); TCA - Taurocholic acid; UV - ultraviolet treatment

Author(s)	Year	Species	Study Type or Endpoint	Copper Effect Concentration (ppb)	Water Source	Water Hardness (ppm)	Hardness based CMC (ppb)	Hardness based CCC (ppb)
Baldwin *et al.*	2003	CS	Reduction in EOG response to L-serine	10	M, F, D	20	2.9	2.3
Baldwin *et al.*	2003	CS	Reduction in EOG response to L-serine	10	M, F, D	120 (artificially raised by $CaCl_2$)	16	10
Baldwin *et al.*	2003	CS	Reduction in EOG response to L-serine	10	M, F, D	240 (artificially raised by $CaCl_2$)	31	19
Baldwin *et al.*	2003	CS	25% (Benchmark) reduction in EOG response to L-serine	2.7 (+ 3.0 background)	M, F, D	20	2.9	2.3
Baldwin *et al.*	2003	CS	25% (Benchmark) reduction in EOG response to TCA	2.3 (+ 3.0 background)	M, F, D	20	2.9	2.3
Baldwin *et al.*	2003	CS	25% (Benchmark) reduction in EOG response to amino acid mixture	3.0 (+ 3.0 background)	M, F, D	20	2.9	2.3
Giattina *et al.*	1982	RT	Copper avoidance threshold for shallow-gradient test (50% reduction)	4.4	Well	28.4	4.1	3.1
Giattina *et al.*	1982	RT	Multiple exposure copper avoidance threshold value (50% reduction)	6.4 (2.6-15.5)	Well	28.4	4.1	3.1

Author(s)	Year	Species	Study Type or Endpoint	Copper Effect Concentration (ppb)	Water Source	Water Hardness (ppm)	Hardness based CMC (ppb)	Hardness based CCC (ppb)
Giattina *et al.*	1982	RT	Single exposure copper avoidance threshold value (50% reduction)	7.3	Well	28.4	4.1	3.1
Hansen *et al.*	1999a	ChS	Copper avoidance	0.8	Well + DW	25	3.6	2.7
Hansen *et al.*	1999a	RT	Copper avoidance	1.6	Well + DW	25	3.6	2.7
Hansen *et al.*	1999b	ChS	50% reduction in EEG response to L-serine	25	Well + DW	25	3.6	2.7
Hansen *et al.*	1999b	RT	50% reduction in EEG response to L-serine	25	Well + DW	25	3.6	2.7
Hansen *et al.*	1999b	ChS	Significant reduction in olfactory receptor cells	50	Well + DW	25	3.6	2.7
Hansen *et al.*	1999b	RT	Significant reduction in olfactory receptor cells	200	Well + DW	25	3.6	2.7
Hara *et al.*	1976	RT	45% reduction in EEG response to L-serine	50 + 20 background	M, D, UV	90	12.2	8.2
Hara *et al.*	1976	RT	Threshold for minimal depression of bulbar response	8 + 20 background	M, D, UV	90	12.2	8.2
Lorz & McPherson	1976	CS	Approximately 16-18 % inhibition of downstream migration following laboratory copper exposure for 165 days	5	Well	89-99	12.0 to 13.3	8.1 to 8.9
Lorz & McPherson	1976	CS	Approximately 28 % inhibition of downstream migration following laboratory copper exposure for 165 days	10	Well	89-99	12.0 to 13.3	8.1 to 8.9

Author(s)	Year	Species	Study Type or Endpoint	Copper Effect Concentration (ppb)	Water Source	Water Hardness (ppm)	Hardness based CMC (ppb)	Hardness based CCC (ppb)
Rehnberg & Schreck	1986	CS	Inhibition of avoidance of L-serine	4.01	Well	30.5	4.4	3.2
Sandahl *et al.*	2004	CS	47% reduction in EOG	10	M, F, D	120	16	10
Sandahl *et al.*	2004	CS	40% reduction in EEG	10	M, F, D	120	16	10
Sandahl *et al.*	2004	CS	20% (Benchmark) reduction in EOG & EEG (combined) for TCA & L-serine (combined)	4.4	M, F, D	120	16	10
Sandahl *et al.*	2004	CS	50% (Benchmark) reduction in EOG & EEG (combined) for TCA & L-serine (combined)	11.1	M, F, D	120	16	10
Sprague	1964	AS	EC_{50} for avoidance	2.3 (+ 2.0 background)	M	18	2.7	2.1